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Development of a Peptide Antibody Specific to Human Glutathione *S*-Transferase Alpha 4-4 (hGSTA4-4) Reveals Preferential Localization in Human Liver Mitochondria

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The reactive cellular products generated during the peroxidation of membrane lipids have been implicated as causative agents in a variety of degenerative diseases and aging. In particular, 4-hydroxynon-2-enal (4HNE) is among the most of the produced during lipid peroxidation. In humans and rodent species, the alpha 4 subclass of glutathione *S*-transferases (mGSTA4-4, rGSTA4-4, hGST-5.8, and hGSTA4-4) exhibits uniquely high glutathione conjugation activity toward 4HNE and other hydroxyalkenals. In human liver, hGSTA4-4-mediated 4HNE conjugation appears to represent the high-affinity pathway for 4HNE detoxification. In the present study, a highly specific polyclonal antibody was developed against hGSTA4-4. Western blotting analysis of human liver subcellular fractions as well as *N*-terminal sequencing revealed that hGSTA4-4 was localized to mitochondrial fractions, but was not detected in cytosolic fractions. Our results provide evidence that in adult liver, hGSTA4-4 is specifically targeted to the mitochondrion to the apparent exclusion of the cytosol. Targeting of hGSTA4-4 to the mitochondrion holds implications for degenerative diseases associated with oxidative stress that arise from aerobic respiration.

Key Words: **peptide antibody; hGSTA4-4; mitochondria; human liver.**

The glutathione *S*-transferases (GSTs;[2] EC 2.5.1.18) constitute a major group of phase II detoxification proteins that protect against a variety of reactive chemicals and products of oxidative stress (1). Currently, seven distinct GST gene families (α, θ, κ, μ, π, σ, and ω) and over 20 distinct soluble GST human proteins have been identified in human liver. With the exception of the microsomal GSTs, the human GST isozymes have been primarily considered to be cytosolic proteins whose conjugates are transported from the cell by ATP-dependent glutathione conjugate efflux proteins (2). In most cases, conjugation of electrophilic compounds through thioether conjugation with glutathione (GSH) reduces cytotoxicity by markedly increasing the compound's water solubility. The range of compounds detoxified by GSTs is remarkably diverse and includes a number of exogenous xenobiotic carcinogens, anticancer drugs, and environmental chemicals. Because a large number of the compounds detoxified by GSTs are highly electrophilic, the level of GST isozyme expression can be an important determinant of sensitivity to chemical toxicity. (reviewed by Hayes and Pulford (1)).

In addition to drug and chemical conjugation, certain GST isozymes can catalyze the reduction of cellular peroxides and can also conjugate endogenous genotoxic α, β-unsaturated aldehydes formed during the peroxidation of membrane lipids (3, 4). In this regard, the GST pathway mitigates the deleterious effects of reactive oxygen species and their toxic products and can thus be viewed as an integral component of the cellular antioxidant defense system. In particular, α, β-unsaturated aldehydes such as 4-hydroxynon-2-enal (4HNE) constitute extremely reactive breakdown products arising from peroxidative reactions (5). The highly reactive and long-lived nature of 4HNE and other such aldehydes allows for diffusion of these compounds

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[2] Abbreviations used: GST, glutathione *S*-transferase; GSH, glutathione; 4HNE, 4-hydroxynon-2-enal; PVDF, polyvinylidene fluoride; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl floride; Mops, 4-morpholinepropanesulfonic acid; Fmoc, 9-fluorenylmethoxycarbonyl; KLH, keyhole limpet hemocyanin; TBS, Tris-buffered saline; HRP, horseradish peroxidase.

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across large cellular distances from the initial site of peroxidation, resulting in both the secondary oxidation and alkylation of cellular proteins and DNA. As a result, 4HNE has been implicated as a causative agent in a number of diseases including cancer (6), cataractogenesis (5, 6), pulmonary inflammation (7), liver disease (8, 9), and athlerosclerosis (10, 11). Because of the cytotoxic nature of 4HNE, it is not surprising that a number of protective biochemical pathways for 4HNE removal, including aldehyde dehydrogenase, aldehyde reductase, and GSTs, are present in mammalian tissues (12).

Of the human GST isozymes, hGSTA4-4 exhibits a uniquely high specific activity toward 4HNE and represents the high affinity pathway for removal of 4HNE in liver (12, 13). Because of their high specific catalytic activity toward such aldehydes and related oxidants, it has been suggested that hGSTA4-4 and its rodent orthologs (mouse GSTA4-4 and rat GSTA4-4) have specifically evolved to protect against oxidative injury *in vivo* (14, 15). However, studies of GST isozyme expression are often limited by the lack of specific antibodies that can clearly discriminate closely related GST isozymes. In the current report, we have analyzed hGSTA4-4 and related protein sequences and incorporated epitope and immunological reactivity analyses to produce a highly specific polyclonal antibody for detection of hGSTA4-4 in human tissues. We further demonstrate that hGSTA4-4 resides in the mitochondrion to the exclusion of the cytoplasm, and therefore may represent a unique subclass of mitochondrial GST. These results may have important implications regarding the role of hGSTA4-4 in the protection against the forms of oxidative stress that occur during mitochondrial respiration processes, as well as during exposure to environmental chemicals that elicit mitochondrial toxicity.

MATERIALS AND METHODS

Chemicals. Cyanogen bromide-activated agarose, protein A, horseradish peroxidase (HRP)-linked rabbit anti-IgY, glutathione, buffer salts, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and from Fisher Scientific (Orlando, FL). Recombinant glutathione *S*-transferases hGSTA1-1, hGSTA2-2, hGSTM1a-1a, hGSTM3-3, hGSTM4-4, mGSTA3-3, mGSTP1-1, rGSTA3-3, and rGSTA5-5 were generously provided by Dr. Theo Bammler of the University of Washington. Recombinant hGSTA4-4 protein (15) was generously provided by Dr. Ann Gustafsson at the University of Uppsala (Sweden). Immobilon-PSQ polyvinylidene difluoride (PVDF) membrane was purchased from Millipore, and Immun-blot PVDF membrane was purchased from Bio-Rad. ECL and ECL Plus detection reagents as well as Hyperfilm MP autoradiographic film were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

Tissue samples and subcellular fractionation. All use of human tissues was approved by the University of Florida Health Center Institutional Review Board. Four human adult liver samples were obtained from the Anatomical Gift Foundation, an independent nonprofit human tissue bank that provides human tissues for biomedical research. The human liver tissues used in this study were from Caucasian male donors (ages 33–63 years) without a prior history of heavy smoking or drug abuse. Isolation of subcellular components from human hepatic tissue was performed as previously described (16). All steps were performed at 4°C. Briefly, liver tissue was minced, placed in 250 mM sucrose, 1 mM EDTA, 0.5 mM DTT, 0.1 mM PMSF, pH 7.5, and homogenized with a Potter-Elvehjem mortar and Teflon pestle. After centrifugation and washing to remove unbroken cells and nuclei, the mitochondria were isolated by centrifugation at 10,000*g* for 10 min. Mitochondrial fractions were then washed twice in isotonic buffer (250 mM sucrose, 2 mM Mops, 1 mM EDTA, 0.5 mM PMSF, pH 7.4) in order to remove adsorbed, nonmitochondrial proteins. Liver cytosolic fractions were isolated by centrifugation at 100,000*g* for 1 h. The microsomal pellets were washed in 250 mM sucrose, 80 mM Tris, 25 mM KCl, 5 mM $MgCl_2$, pH 8.0, and recentrifuged at 100,000*g* for 60 min. The microsomal pellets were resuspended in 100 mM Tris, 1 mM EDTA containing 20% glycerol, pH 8.0. All cellular fractions were stored at −80°C until use. The protein content of samples was determined using the bicinchoninic acid method (17).

Production of an anti-hGSTA4-4 peptide antibody and selection of hGSTA4-4 IgY. Protein sequences for the known alpha class human GST subunits, including hGSTA1 (Accession No. S49975), hGSTA2 (NM_000846), hGSTA3 (AF020919), and hGSTA4 (AAD27706) were aligned using Clustal W (http://bioweb.pasteur.fr/seqanal/interfaces/clustalw.html (18)), and analyzed for areas of sequence divergence. Of these human alpha class GSTs, hGSTA1, hGSTA2, and hGSTA3 are 90% identical, whereas hGSTA4 diverges with 53% identity relative to the other three alpha class GSTs (19). Once identified, the divergent regions of these peptides were analyzed for theoretical secondary structure using NNPredict (20). The amino acid sequence was also analyzed for antigenicity through the use of the algorithm of Hopp and Woods (21) and through inspection of charge distribution. The candidate peptides were placed in the context of the high-resolution crystallographic structure of hGSTA4-4 (Protein Database No. 1GUL) (22)), and the oligopeptide Ac-MHPFLKPDDQQKEVVN-NH_2 was chosen as a divergent and antigenic sequence. This peptide was commercially synthesized (American Peptide Company, Sunnyvale, CA) using the 9-fluorenylmethoxycarbonyl (Fmoc) strategy of Wellings and Atherton (23). A C-terminal cysteine was added to the native sequence to allow for conjugation to the carrier protein keyhole limpet hemocyanin (KLH). The amino and carboxyl termini were acetylated and amidated, respectively, in order to shield these charged and potentially antigenic groups, since they are not present in the native protein. The KLH–peptide conjugate was injected into chickens (Strategic BioSolutions, Ramona, CA), and IgY was purified from the harvested eggs. In order to remove IgY cross-reactivity, the purified yolk antibodies were further selected by affinity chromatography. Specific recombinant human GSTs were conjugated to cyanogen bromide-activated agarose beads (Sigma Chemical Co.) according to the manufacturer's instructions. For positive selection, recombinant hGSTA4-4 was used as a conjugate. Excess conjugate was combined with purified, polyclonal IgY and the mixture was incubated for 2 h at 23°C. This was eluted from the rinsed matrix of a spin column by the addition of 0.1 M glycine, pH 2.8, followed by centrifugation at 500*g* for 1 min. The eluate was neutralized by the addition of 0.1 M Tris–HCl, pH 8.9, and this solution was dialyzed for 40 h at 4°C against 1000 vol of TBS (20 mM Tris, 0.8% NaCl, pH 7.6). For negative selection, the positively selected IgY was incubated with an excess of recombinant hGSTM1a-1a agarose conjugate. The flow-through from this step was collected and used in subsequent Western blotting experiments.

SDS–PAGE and Western blotting analysis. Human liver subcellular proteins were electrophoretically separated and transferred to PVDF membrane. Specific quantities and conditions are described in the figure legends. For Western blotting analysis of recombinant proteins, the polyclonal anti-hGSTA4 peptide antibody was used at a

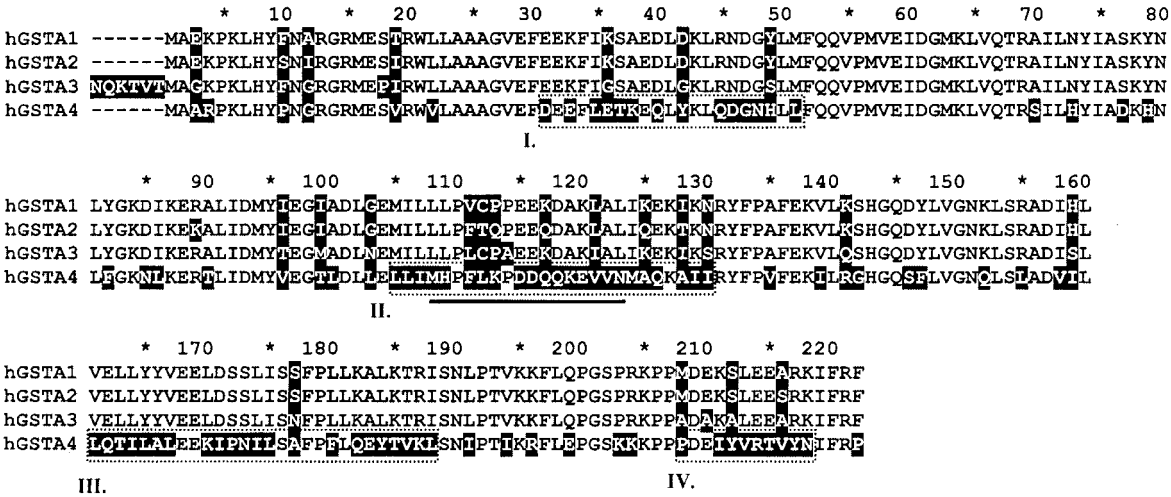

FIG. 1. Protein sequence alignment of alpha class human GST subunits hGSTA1, hGSTA2, hGSTA3, and hGSTA4. Highlighted lettering represents nonconserved amino acid residues. Boxed sequences indicate the four regions of greatest divergence in hGSTA4-4 relative to other human alpha class GSTs. The underlined sequence indicates the oligopeptide from the hGSTA4 sequence used to generate the anti-hGSTA4-4 peptide antibody.

dilution of 1:2000 and the secondary antibody (rabbit anti-chicken IgY conjugated to horseradish peroxidase, HRP) was used at a dilution of 1:10,000 with ECL as the detection reagent. Preabsorption of cross-reactive IgY was performed using excess (100 μg) recombinant hGSTM1a-1a per 25 μl undiluted primary antisera. For Western blotting analysis of subcellular fractions, the selected IgY was used at a dilution of 1:10,000 and the secondary antibody was used at a dilution of 1:200,000, with ECL Plus as the detection reagent. Densitometric analysis of hGSTA4-4 protein expression was performed using Bio-Rad Quantity One™ software.

Analysis of subcellular marker enzymatic activities. In order to determine the extent of cross-contamination of subcellular preparations, marker enzymes specific for mitochondria, microsomes, and cytosol were measured in subcellular fractions prepared from six adult liver tissues. Cytochrome c oxidase activity (a mitochondrial marker, 24–26), NADPH-cytochrome c reductase (a microsomal marker, 16, 27, 28), and lactate dehydrogenase (a cytosolic marker, 29), were all measured as described with the exception that assay volumes were modified such that the analyses could be performed in a microplate format.

Immunoprecipitation and N-terminal sequencing of mitochondrial protein. Approximately 1 ml (8 mg) of an adult liver mitochondrial preparation was washed at 4°C in an isotonic buffer (250 mM sucrose, 2 mM Mops, 1 mM EDTA, 0.5 mM PMSF, pH 7.4) and collected by centrifugation at 10,000*g* for 10 min at 4°C. The pellet was suspended in 500 μl ice-cold TBS containing 1 mM PMSF. Sodium deoxycholate (0.1%) and 25 μg of selected IgY was added to the mitochondrial suspension followed by end-over-end, overnight mixing at 4°C. Twenty micrograms of rabbit anti-chicken IgY was then added, followed by a 2-h incubation. Insoluble material was separated from solubilized protein by brief centrifugation at 500*g* and the supernatant was transferred to a clean tube. Fifty microliters of a protein A–agarose slurry (Sigma) was added, and the mixture was further incubated for 2 h. The mixture was then placed in a centrifuge column, and the agarose beads were washed three times with 500 μl TBS/PMSF/deoxycholate, followed by three washings with 500 μl TBS/PMSF and a brief centrifugation at 500*g*. Bound proteins were removed from the protein A–agarose beads by addition of 100 μl of dissociation buffer consisting of 0.5 M Tris–HCl, pH 6.8, 5% glycerol, 1% SDS, and 0.001% bromophenol blue. The mixture was incubated for 3 min at 95°C, and the eluate was recovered from the column. Reducing agents were specifically omitted from the dissociation buffer in order to prevent light chains of immunoglobulins from comigrating with the proteins of interest, which in theory have a similar molecular weight to light chains. The eluate was directly subjected to electrophoresis and subsequently transferred to Immobilon-PSQ PVDF membrane in 10 mM Mes, pH 6.0, and 20% methanol, overnight at 20 V. In order to avoid N-terminal blocking, the membrane was only lightly stained (0.02% Coomassie R-250, 40% methanol, 5% acetic acid) for 30 s and immediately destained for 1 min. The membrane was rinsed with three changes of distilled H_2O for 5 min and then allowed to air dry. A single band was excised and submitted for N-terminal sequence analysis (30).

RESULTS

Sequence Alignment and Antibody Production

In order to avoid cross-reactivity of novel immunological reagents for hGSTA4-4, peptide sequences of the alpha class GSTs were compared through an alignment (Fig. 1). Four regions were identified in the hGSTA4 sequence that showed significant divergence from the other members of the alpha class GSTs. These regions from the hGSTA4 sequence are as follows: I, Asp-31 → Leu-51; II, Leu-109 → Ile-134; III, Leu-165 → Leu-192; IV, Pro-212 → Asn-222. These peptide sequences were analyzed for theoretical secondary structure (20) and were compared to the crystallographic structure (Fig. 2A (22)). Peptide regions that exhibited a high degree of similarity between theoretical secondary structure and crystallographic structure were considered more favorably than those regions that did not have this characteristic. The distribution of charge and hydrophobicity, which strongly contribute to antigenicity, were considered (Fig. 2A), as was the antigenicity index generated by the Hopp and Woods algorithm (Fig. 2B (21)). Based on these factors,

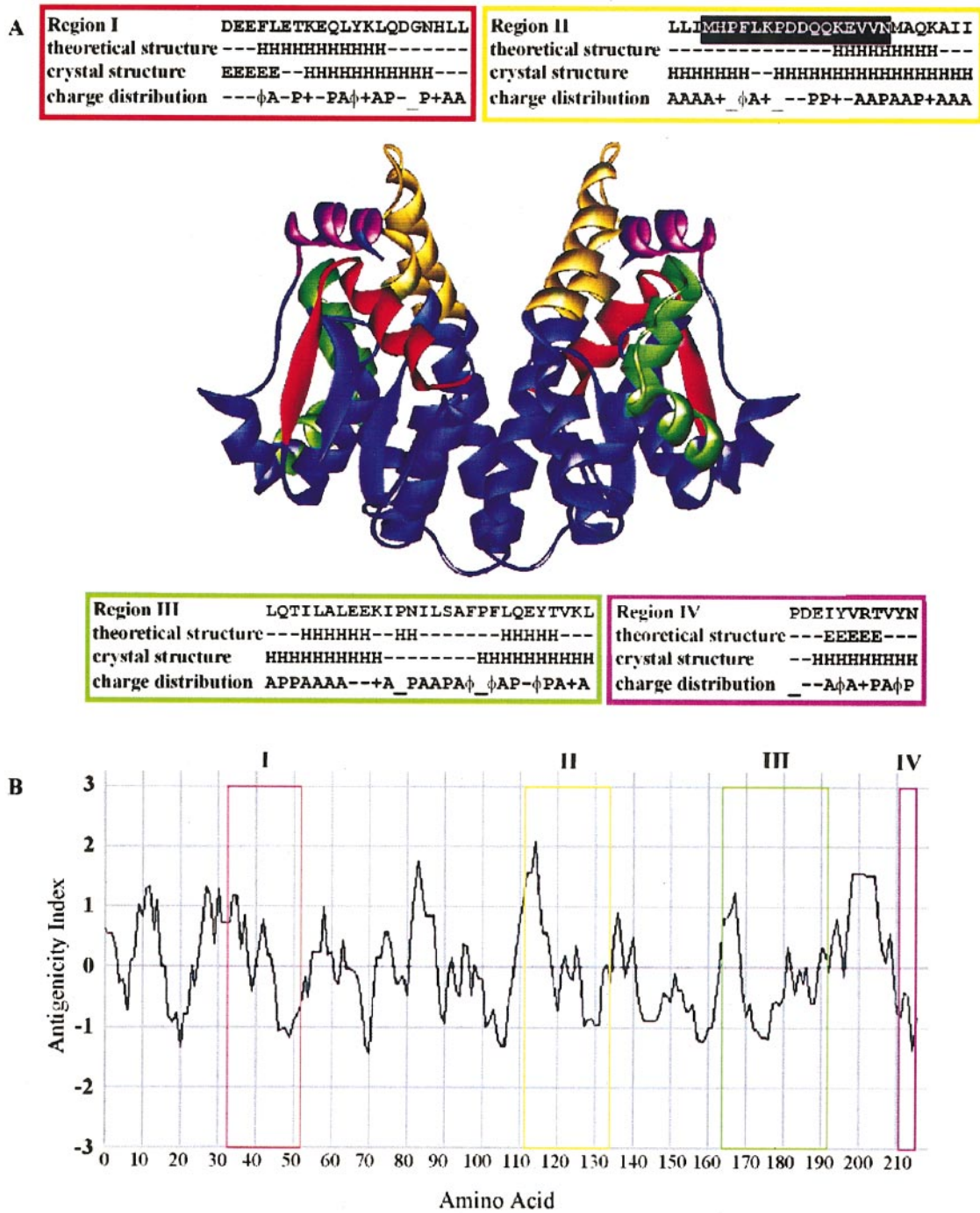

FIG. 2. Structural criteria for determining the useful peptide sequences for hGSTA4-4 antibody production. (A) The four most divergent regions of the hGSTA4 sequence are listed with their theoretical secondary structure, crystal structure (E, beta sheet; H, helical), and charge distribution (A, aliphatic; P, polar; +, positively charged; −, negatively charged; ϕ, aromatic; _, none). The high-resolution crystallographic structure of hGSTA4-4 is provided (22). Portions of the homodimer are color-coded according to the four represented regions: region I, red; region II, yellow; region III, green; region IV, violet. The oligopeptide sequence used in this study is highlighted in black. (B) The four color-coded regions are shown superimposed against a Hopp and Woods (21) antigenicity plot where potential antigenicity is given as a function of the hGSTA4 polypeptide sequence.

as well as the steric accessibility of the region in the context of the native enzyme, region II was chosen as a template for the production of a synthetic peptide. The 16-mer oligopeptide Met-112 → Asn-127 was selected from this region. This 16-mer exhibited less than 13% identity relative to the corresponding hGSTA1 peptide,

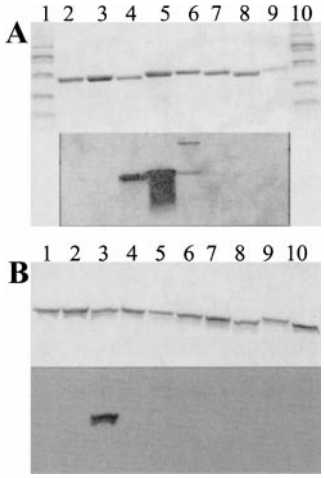

FIG. 3. Cross-reactivity of the anti-hGSTA4-4 antibody against recombinant GSTs. Three micrograms of purified human and rodent GST proteins were resolved on a 12% polyacrylamide gel by SDS–PAGE and transferred to a PVDF membrane. Immunoblot analysis was performed using the anti-hGSTA4-4 peptide antibody before (A) and after (B) preabsorption of antibody using recombinant hGSTM1a-1a. In each panel the immunoblot, stained for total protein, is presented above the resulting autoradiogram. The GST subunits in A are hGSTA1-1 (lane 2), hGSTA2-2 (lane 3), hGSTA4-4 (lane 4), hGSTM1a-1a (lane 5), hGSTM3-3 (lane 6), mGSTA3-3 (lane 7), mGSTP1-1 (lane 8), and rGSTA5-5 (lane 9). Lanes 1 and 10 are molecular weight markers. The GST subunits in B are hGSTA1-1 (lane 1), hGSTA2-2 (lane 2), hGSTA4-4 (lane 3), hGSTM1a-1a (lane 4), hGSTM3-3 (lane 5), hGSTM4-4 (lane 6), mGSTA3-3 (lane 7), mGSTP1-1 (lane 8), rGSTA3-3 (lane 9), and rGSTA5-5 (lane 10).

like the native sequence, was largely alpha helical in secondary structure and exhibited a relatively high degree of antigenicity. According to the crystallographic data, region II resides on the outside of the dimer, thus allowing approach of an immunoglobulin. Moreover, in an ungapped, low-complexity search, the 16-mer did not match any other sequence in the BLAST database. Rapid and humane production of antibodies was possible through the use of chicken as the host species, as relevant immunoglobulins may be purified directly from egg yolk (31). An injection of 1.5 mg of the synthesized peptide resulted in the production of crude sera yielding an average titer of 1:70,000 against the peptide as compared to 1:50 for the preimmune sera, determined by an enzyme-linked immunosorbent assay (data not shown).

Specificity of the hGSTA4-4 Peptide Antibody

The specificity of the hGSTA4-4 peptide antibody was determined by immunoblot analysis (Fig. 3). The chicken antisera did not react with the other alpha class GSTs that were present. However, the antisera displayed cross-reactivity with the recombinant hGSTM1a-1a protein, as observed in Fig. 3A. The use of preimmune sera in subsequent immunoblot analysis resulted in the specific recognition of hGSTM1a-1a, but not hGSTA4-4, indicating that the IgY cross-reactivity was due to antibodies that were present prior to immunization with the oligopeptide. Preabsorption of the IgY antibody with recombinant hGSTM1a-1a resulted in a marked loss of cross-reactivity to hGSTM1a-1a (Fig. 3B). Similarly, selection of the antisera through affinity chromatography produced a loss of cross-reactivity as determined by Western analysis (data not shown).

Subcellular Localization of hGSTA4-4 in Human Liver

In preliminary Western blotting analyses, we were unable to detect hGSTA4-4 protein in human liver cytosol. In order to explore the possibility that hGSTA4-4 is a noncytosolic GST, cytosolic and mitochondrial fractions were prepared from total human liver. Western analysis of these subcellular fractions demonstrated the presence of hGSTA4-4 in mitochondria but not cytosolic fractions (Fig. 4). The cross-reacting protein, however, showed an altered electrophoretic mobility of approximately 29–30 kDa, making it approximately 4 kDa greater than the recombinant enzyme. Also, a very faint band with similar mobility characteristics as the recombinant hGSTA4-4 protein was present in both crude homogenate and mitochondria, but was not seen in cytosol (Fig. 4). The 30-kDa band that corresponded to hGSTA4-4 was consistently of higher molecular weight than the recombinant form by approximately 3–4 kDa (Fig. 4), which brought its identity into question. To confirm the identity of the 30-kDa band, mitochondria were subjected to immunoprecipitation and the resulting immunoprecipitate was electrophoretically separated by SDS–PAGE and transferred to PVDF (Fig. 5). The 30-kDa band was excised and subjected to N-terminal sequencing. The observed sequence, AARPKLHYPNG, was identical to the hGSTA4 N-terminus (Fig. 1), and in an ungapped search did not match any other sequence retrieved in the BLAST database.

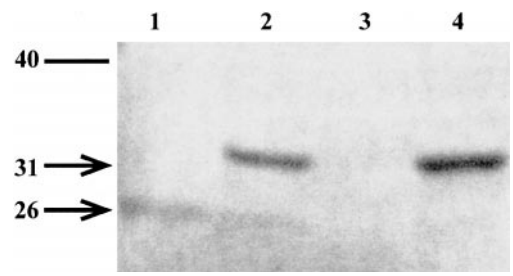

FIG. 4. Western analysis of hGSTA4-4 in cytosolic and mitochondrial fractions from human liver. Protein samples were applied as follows: 0.4 μg recombinant hGSTA4-4 (lane 1), 62 μg each for crude homogenate (lane 2), cytosol (lane 3), and mitochondria (lane 4).

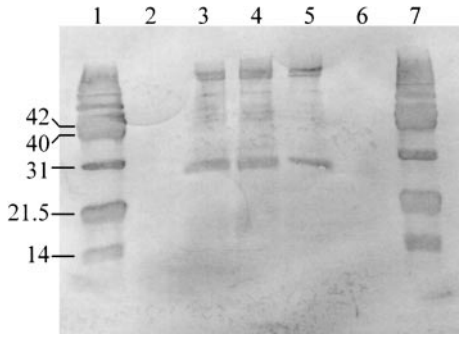

FIG. 5. Coomassie staining of immunoprecipitates from human liver mitochondria after SDS–PAGE on 15% polyacrylamide and transfer to PVDF membrane. Lanes 1 and 7 are molecular weight markers. Lanes 3 through 5 represent purified mitochondrial immunoprecipitates.

As a means of verifying the subcellular localization of hGSTA4-4, a second immunoblot was constructed using proteins from initial homogenate, as well as from mitochondrial, microsomal, and cytosolic fractions. As observed in Fig. 6A, the most prominent hGSTA4-4 band was associated with the mitochondrial fraction, followed by the crude homogenate. A very faint band cross-reacting to the hGSTA4-4 antibody was observed in the microsomal fraction, whereas there was no cross-reactivity observed in the cytosolic fraction (Fig. 6A). The relative purity of the subcellular fractions was then determined using marker enzymes specific to the mitochondrial, microsomal, and cytosolic fractions. The specific activity of cytochrome c oxidase in the microsomal and cytosolic fractions was 35 and 1% that of the mitochondria, respectively (Fig. 6B). NADPH-cytochrome c reductase activities in mitochondria and cytosol were 30 and 10% that of the microsomal fractions. In addition, lactate dehydrogenase activities in mitochondrial and microsomal fractions were 22 and 18% of that observed in the cytosolic fractions (Fig. 6B). Relative intensities of hGSTA4-4 protein bands from Fig. 6A were normalized through pixel densities per milligram protein to that of the most prominent band from the subcellular fractions, which in this case was the mitochondrial fraction. These percentage values were compared directly to the relative specific activities of the marker enzymes. As observed in Fig. 6B, the hGSTA4-4 protein levels in the subcellular fractions were highly correlated with mitochondrial cytochrome c oxidase activities.

DISCUSSION

Previous studies using polyclonal GST antibodies have shown considerable cross-reactivity with nontarget GST proteins, resulting in experimental limitations in the analysis of GST isozyme expression (32–35). Such cross-reactivity among GST antibodies can make discrimination of closely related proteins within the various classes particularly difficult. For example, alpha class GST subunits hGSTA1 and hGSTA2 share 92% sequence identity and co-occur in human tissues. Although both of these enzymes display remarkably high activities toward fatty acid hydroperoxides and phospholipid hydroperoxides, they differ in their kinetic affinities and activities toward these substrates (36). The high sequence homology among hGSTA1 and hGSTA2 subunits has made it difficult to produce antibodies capable of discriminating the dimeric products of these two alpha class GST isozymes (36, 37). While the hGSTA4 protein exhibits lowered identity relative to hGSTA1 and hGSTA2 proteins, use of the complete hGSTA4-4 enzyme for immunization would have posed considerable risk of generating a nonspecific immunoglobulin.

In the present study, we report the development of a polyclonal peptide antibody that is specifically designed to recognize hGSTA4-4 to the exclusion of other

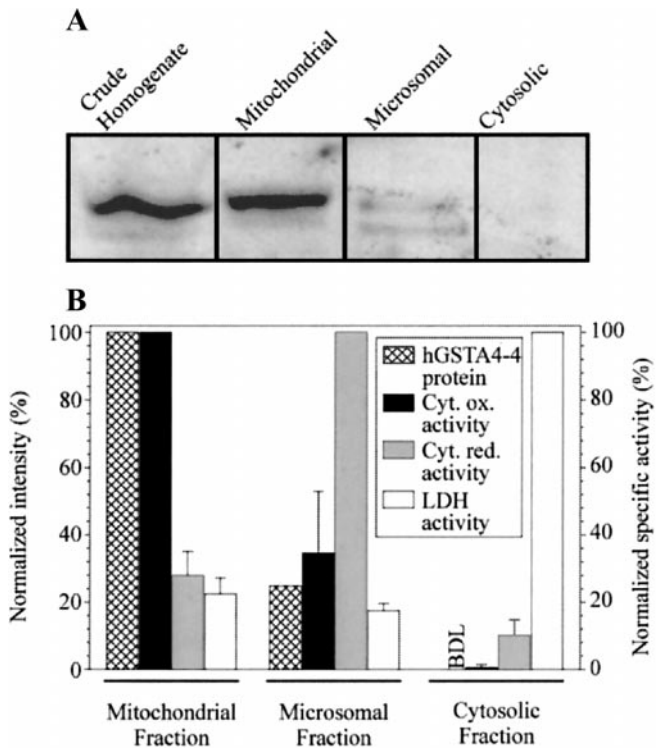

FIG. 6. Colocalization of subcellular marker enzymes with hGSTA4-4 protein. (A) Western analysis using anti-hGSTA4-4 antibody against subcellular fractions from human adult liver tissue. (B) Comparison of relative specific activities of enzymatic markers and staining intensity from Western analysis. Values were scaled to the highest value from each data set and compared through a percentage. Mean ± SD activities for the marker enzyme activities were as follows, mitochondrial cytochrome c oxidase (Cyt. ox.), 15 ± 5.4 μmol/min/mg; microsomal NADPH cytochrome c reductase (Cyt. red.), 43 ± 21 μmol/min/mg; and cytosolic lactate dehydrogenase (LDH), 1110 ± 97 μmol/min/mg.

closely related GSTs. Our approach was similar to that used by other groups that have developed cytochrome P-450 isozyme-specific peptide antibodies (38). We initially identified peptide regions from hGSTA4-4 that showed the highest degree of divergence from the three closest related human alpha class GST proteins (e.g., hGSTA1, hGSTA2, and hGSTA3). The regions of highest divergence were then analyzed for peptide secondary structure. In order to maximize the odds of developing a polyclonal antibody that would possess a high affinity for the native enzyme, charge distribution, potential antigenicity, and steric accessibility were all considered during final peptide selection. This approach led to the utilization of amino acids Met-112 through Asn-127 for the production of a synthetic oligopeptide. This peptide region was highly divergent, exhibited high potential antigenicity, and resided on the exterior of the crystal structure. Moreover, this region contained residues Met-112 and Phe-115, which contribute to the H-site of the enzyme (22). The oligopeptide resulted in the production of a highly specific polyclonal antibody for hGSTA4-4. The initial cross-reactivity of the antiserum to hGSTM1a-1a, while not predicted, was easily removed by immunoaffinity chromatography.

Through a combination of Western blotting analysis, immunoprecipitation and N-terminal sequencing, we provide evidence that hGSTA4-4 localizes to the mitochondrion but is not found in the cytosol. We did not predict that hGSTA4-4 may be mitochondrial, as the overwhelming majority of mammalian GST isozymes are reported to be cytosolic, or to a lesser extent, microsomal (2). However, a review of the GST literature revealed three reports providing precedence for mitochondrial GST (33, 39, 40). In particular, Bhagwat *et al.* demonstrated that rGSTA4-4, the rat ortholog of hGSTA4-4, is found in both mitochondrial and cytosolic fractions prepared from rat lung, brain, and liver (39). In addition, GST alpha-like proteins have been reported in mouse, rat, and dog liver mitochondria (33). However, the aforementioned study did not conclusively identify the particular alpha GST subtypes present in the mitochondrial fractions analyzed. In general, most proteins that are targeted to the mitochondrion are translated as presequences with N-terminal targeting leaders which are typically cleaved from the mature protein after targeting is complete (41). If hGSTA4-4 is indeed mitochondrial, as our results indicate, it would be among a less common group of mitochondrial proteins with a cryptic targeting sequence. Thus, while overt sequence characteristics do not support mitochondrial targeting of GSTA4-4 proteins, our observations coupled with the demonstrated mitochondrial localization of rGSTA4-4, strongly suggest that GSTA4 enzymes may have specifically evolved for mitochondrial detoxification. It is possible that a portion of the hGSTA4-4 sequence which diverges from other human alpha class GSTs serves to target hGSTA4-4 to the mitochondrion.

The endogenous hGSTA4-4 protein detected by our antibody exhibited an altered electrophoretic mobility relative to the recombinant protein, as evinced by an approximate 4-kDa size difference. This apparent change in molecular weight may have resulted from the existence of a novel hGSTA4-4-like 30-kDa GST, from alternative *hGSTA4* mRNA splicing or by a post-translational modification of the hGSTA4-4 protein. It is possible that a novel protein with high homology to hGSTA4-4, but exhibiting greater molecular weight, may be cross-reacting with the hGSTA4-4 antibody. If this were the case, however, one would have expected to observe two prominent bands through Western analysis or immunoprecipitation, with one band constituting hGSTA4-4 and the other band representing the related 30-kDa protein. Also, neither the reported structure of the hGSTA4 gene nor primer extension analysis (19) supports alternative splicing of the *hGSTA4* transcript that would ultimately account for the production of a higher molecular weight protein. These findings lend credence to the possibility that the alteration in electrophoretic mobility may have been the result of protein modification. The very faint GST-like bands in the crude homogenate and mitochondrial lanes of Figs. 4A and 5 that comigrate with recombinant hGSTA4-4 may be indicative of an hGSTA4-4 which is devoid of posttranslational modification. Forthcoming investigations may shed light on these questions.

Mitochondria are under constant oxidative challenge from the continuous production of reactive oxygen species during oxidative phosphorylation. In this regard, mitochondrial DNA typically exhibits 10–23 times higher levels of oxidized bases per microgram than does nuclear DNA (42). Mitochondrial lipids are under constant attack from reactive oxygen such as O_2^-, H_2O_2, and $^{\cdot}OH$ (43). These cellular oxidants catalyze the autooxidation of membrane lipids and the accumulation of malondialdehyde and 4HNE. Ultimately, the loss of membrane integrity can occur along with alkylation of proteins and DNA. Our observation that hGSTA4-4 appears to reside in the mitochondria is suggestive of a role for hGSTA4-4 in maintaining favorable mitochondrial redox status during cellular respiration.

The characteristic substrate specificity profile and mitochondrial localization of human hGSTA4-4 suggests that the expression of this enzyme may be a determinant of susceptibility to oxidative injury and degenerative disease states in which a loss of mitochondrial function has been noted (43). In this regard, other alpha class GSTs show high interindividual variation in human liver, although the genetic basis for this variation remains unclear (44). Other GST

isozymes have been shown to exhibit polymorphic expression in humans, and molecular epidemiological studies have demonstrated an association with polymorphic expression of GST isozymes and susceptibility to certain diseases (2). For example, GSTM1 exhibits polymorphisms that arise from homo- and heterozygotic combinations of the *GSTM1*0*, *GSTM1*A*, and *GSTM1*B* alleles. *GSTM1*0* represents the gene deletion, and homozygotes (null phenotype) express no protein, while *GSTM1*A* and *GSTM1*B* differ by a single base in exon 7. Homozygosity at *GSTM1*0* is associated with an increased risk of various malignancies, including lung cancer (45–47). In addition, genetic polymorphisms at the hGSTP1 locus have recently been associated with increased susceptibility to bladder, testicular, and prostate cancer (48).

In conclusion, we have developed a novel immunological reagent for specifically determining the presence of hGSTA4-4 in human tissues. Furthermore, we have shown that in human liver, hGSTA4-4 is localized in mitochondrial fractions to the apparent exclusion of the cytosol. Currently, we are characterizing potential interindividual differences in mitochondrial hGSTA4-4 expression toward an understanding of the role of mitochondrial GST expression in susceptibility to oxidative injury.

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